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(54) Title: STRESS TOLERANT YEAST MUTANTS

(57) Abstract

The invention provides methods and compositions relating to stress tolerant yeasts; in particular, yeast mutants deficient in the expression of functional ATH1 gene product (Ath1p). Such yeast have enhanced tolerance to dehydration and freezing, are able to grow to a higher cell density over a range of fermentable carbon source concentrations, are able to produce and/or tolerate higher levels of ethanol and trehalose. Nucleic acids comprising ATH1 gene sequences are used in hybridization probes and PCR primers, in expression vectors, etc. The invention provides methods for producing a yeast mutant with improved survival ability under stress conditions which involve identifying mutations disrupting ATH1 expression using Ath1-specific reagents or ATH1 hybridization probes or primers.

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STRESS TOLERANT YEAST MUTANTS

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INTRODUCTION

Technical Field

The technical field of this invention concerns a specific genetic mutation in yeast which provides enhanced stress tolerance.

Background

The non-reducing disaccharide *O*-α-D-glucopyranosyl-1—>1-α-D-glucopyranoside, commonly known as trehalose, was discovered in 1832 (Wiggers, 1832) in a fungus, *Secale cornutum*. Since then, trehalose has been found in a wide variety of organisms including additional fungi, bacteria, plants, insects and other invertebrates. In *Saccharomyces cerevisiae*, trehalose is one of the major storage carbohydrates, accounting for up to 23% or more of the dry weight of the cells, depending on growth conditions and the stage of life cycle (Elbein, 1974).

Trehalose is believed to function in yeast as an energy source in spore germination and as a protecting agent for maintaining structural integrity under environmental stresses such as heat and desiccation (Thevelein, 1984). More recent results, however, indicate that the bulk of trehalose accumulated in yeast under mild heat treatment is not sufficient to account for the acquisition of thermotolerance (Arguelles, 1994; Nwaka et al., 1994; Winkler et al., 1991). The concentration of trehalose in the yeast cell is the result of the activities of the synthesizing bifunctional enzyme trehalose-6-phosphate-synthase/trehalose-6-phosphate phosphatase (Vuorio et al., 1993) and the trehalose hydrolyzing enzymes, *e.g.* cytosolic neutral trehalase (App and Holzer, 1989) and vacuolar acid trehalase (Mittenbühler and Holzer, 1988). The recently cloned neutral trehalase (NTH) is considered to be the key enzyme responsible for trehalose degradation

in intact yeast cells (Kopp et al., 1993; Wiemken, 1990), however, very little is known about the biological function and possible control mechanisms for vacuolar acid trehalase (ATH). ATH has been shown to be glycosylated (Londesborough and Varimo, 1984; Mittenbühler and Holzer, 1988) and activation is dependent on the *PEP4* gene product, proteinase A (Harris and Cotter, 1987). The physiological role of ATH and the coordination of its function with that of NTH is unknown.

Due to its role in stress protection, trehalose has important commercial applications for the baking and brewing industries (Mansure et al., 1994; Oda et al., 1986; Hino et al., 1990; Gelinas et al., 1989). The synthesis and degradation of trehalose is important in yeast cell physiology at various stages of growth; mobilization of trehalose and the timing of its metabolism are critical for yeast growth and survival.

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to stress tolerant yeast; in particular, yeast mutants deficient in the expression of functional ATH1 gene product (Ath1p). An exemplary haploid *S. cerevisiae* strain, identified as MDY3, is deposited at the Section of Microbiology, University of California, Davis.

Such yeast have broad industrial application. For example, in the baking industries, the enhanced tolerance to dehydration and freezing make the mutant yeast particularly suited for use in frozen dough and dehydrated yeast products. In brewing, the mutant yeast strains are able to grow to a higher cell density over a range of fermentable carbon source (e.g. glucose) concentrations and are able to produce and/or tolerate higher levels of ethanol. Accordingly, these strains are used to generate higher ethanol concentrations, take fermentation to a greater degree of completion (to make drier wine) and complete fermentation faster. The subject yeast also find use as an improved source of trehalose (trehalose is used commercially as a protectant in food and pharmaceutical processes) and as a source of ethanol as fuel or additive for spirits: e.g. using inexpensive fermentation substrates such as molasses or corn syrup.

The invention also encompasses isolated nucleic acids comprising ATH1 (SEQUENCE ID NO:1) or fragment thereof capable of hybridizing under stringent conditions with ATH1; and in particular, genetic constructs comprising in 5' - 3' orientation, a first ATH1 fragment capable of hybridizing under stringent conditions with ATH1, an intervening sequence, and a second different ATH1 fragment capable of hybridizing under stringent conditions with ATH1.

The invention provides methods for producing a yeast mutant with improved survival ability under stress conditions which involve identifying mutations disrupting ATH1 expression using Ath1-specific reagents or ATH1 hybridization probes or primers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows growth versus percent survival over time following dehydration curves.

Figure 2 shows growth versus percent ethanol over glucose concentration curves.

Figure 3 shows growth versus trehalose concentration over glucose concentration curves.

Figure 4A shows growth versus percent survival over time following dehydration curves.

Figure 4B shows percent survival versus trehalose concentration over time following dehydration curves for ATH1 mutant yeast.

Figure 4C shows percent survival versus trehalose concentration over time following dehydration curves for wild-type yeast.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The subject yeast mutants are specifically deficient in the expression of a functional ATH1 gene product. Such a mutant expresses less than half, preferably less than 25%, more preferably less than 10% and more preferably less than 1% of the functional Ath1p expressed by the corresponding wild-type yeast. A variety of genetic mutations yield mutants deficient in the expression of functional ATH1

gene product; preferred mutants have at least one ATH1 allele rendered non-functional (i.e. incapable of generating a functional ATH1 gene product). In one embodiment, the invention provides such mutants wherein said mutant or an ancestor of said mutant was generated by genetically engineering a yeast cell to create a nonfunctional mutation in an Ath1p allele of said yeast cell. A particular exemplary mutant, known herein as MDY3, is on deposit at the University of California, Davis, Section of Microbiology.

Phenotypically, such yeast mutants share a number of characteristics including enhanced survival following dehydration, enhanced ethanol tolerance, and enhanced trehalose production. The post-dehydration survivability enhancement is most apparent as the yeast transit from exponential to stationary growth phases. For cells experiencing dehydration at time points from about 0-10 hours following that transition, and usually from about 1-5 hours following transition, until about 20, often 30, and even 40 or more hours later, the mutant yeast demonstrate a significant enhancement in survivability as compared with the corresponding wild-type yeast. See for example, Figure 1 and Figure 4 A. This enhanced survivability often correlates with enhanced trehalose concentrations; see, Figures 4B and 4C. Mutant survival is generally at least 10%, preferably at least 20%, more preferably at least 50%, more preferabiy at least 100% (i.e. double) more than the corresponding wild-type yeast during at least one time point.

The subject mutants demonstrate enhanced ethanol tolerance. The mutant yeast are thus able to generate higher ethanol media concentrations than their wild-type counterparts: generally at least 5%, preferably at least 10%, more preferably at least 20% higher ethanol concentration at least one nutrient condition and time point. See for example, Figure 2. The subject mutants also demonstrate enhanced trehalose concentrations as compared with their wild-type counterparts: generally at least 5%, preferably at least 10%, more preferably at least 20% higher trehalose concentration at least one nutrient condition and time point. See for example, Figure 3.

The invention provides isolated nucleic acids comprising ATH1 (SEQUENCE ID NO:1) or fragments thereof capable of hybridizing under

stringent conditions with ATH1. The subject nucleic acids are either isolated, partially purified, or recombinant. An "isolated" nucleic acid is present as other than a naturally occurring chromosome or transcript in its natural state and isolated from (not joined in sequence to) at least one nucleotide with which it is normally associated on a natural chromosome; a partially pure nucleic acid constitutes at least about 5%, preferably at least about 30%, and more preferably at least about 90% by weight of total nucleic acid present in a given fraction; and a recombinant nucleic acid is flanked - joined in sequence on at least one side - by at least one nucleotide with which it is not normally associated on a natural chromosome.

The subject nucleic acids include ATH1 probes and primers comprising one or more ATH1 fragments capable of hybridizing with ATH1 under stringent conditions, e.g. under stringency conditions characterized by a hybridization buffer comprising 0% formamide in 0.9 M saline/0.09 M sodium citrate (SSC) buffer at a temperature of 37°C and remaining bound when subject to washing at 42°C with the SSC buffer at 37°C. Preferred nucleic acids will hybridize in a hybridization buffer comprising 20% formamide in 0.9 M saline/0.09 M sodium citrate (SSC) buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 2 X SSC buffer at 42°C.

The subject nucleic acids may be introduced into a variety of genetic constructs, plasmids, vectors and cells. For example, a construct useful in generating ATH1 deletion mutants comprises in 5′ - 3′ orientation, a first ATH1 fragment thereof capable of hybridizing under stringent conditions with ATH1, an intervening sequence, and a second different ATH1 fragment thereof capable of hybridizing under stringent conditions with ATH1.

The invention also provides ATH1 gene products and ATH1 gene product-specific binding agents. ATH1 gene products include ATH1 translation products such as Ath1p (SEQUENCE ID NO:2). Binding agents specific for such gene products are produced or identified by a variety of ways. For example, Ath1p peptides are used as immunogens to generate specific polyclonal or monoclonal antibodies. See, Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, for general methods. Other prospective Ath1p-peptide

specific agents are screened from large libraries of synthetic or natural compounds using any convenient binding assay. Such binding agents are capable of binding an ATH1 gene product with an equilibrium constant at least about 10^7 M^{-1}, preferably at least about 10^8 M^{-1}, more preferably at least about 10^9 M^{-1}.

The invention provide methods for producing ATH1 yeast mutants with improved survival ability under stress conditions. In general, the methods involve subjecting a population of yeast to stress conditions, detecting in said population a yeast mutant deficient in the expression of functional Ath1p gene product and growing said yeast mutant to obtain yeast with improved survival ability under stress conditions. The methods may include subjecting the yeast population to conditions promoting mutation, which may be random (e.g. chemical, uv radiation, etc.) or site-directed mutagenesis conditions, of one or more ATH1 alleles and selection for the mutant genotype. The stress conditions provide a selective growth and/or survival advantage for ATH1 mutants deficient in the expression of functional Ath1p gene product (e.g. elevated ethanol media concentration, dehydration, etc.). Targeted mutations are conveniently detected using ATH1 specific oligonucleotide primers or probes, by using Ath1p gene product-specific binding agents (i.e. detecting a deficiency in the expression of functional ATH1 gene product) such as Ath1p specific antibodies, or any other convention method.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Protocol for Figure 1

Examination of effect of Δath1 mutation on survival following dehydration.

1. Grow SEY6210 or SEY6210 Δath1::URA3 in YNBD medium or YNBD medium -URA. Subculture into YPD (2% glucose) and monitor growth.
2. Spin 4 x 5 O.D.s at 14 hour time point and wash in 50 mM MES pH 5.5. Resuspend in 0.45 ml 50 mM MES pH 5.5.
3. Transfer 0.1 ml to two new microfuge tubes and hold at 24°C.
4. Read O.D.600 of remaining sample (25 ul in 2 ml).

5. Allow one set of samples to dehydrate in speed vac to constant weight (9 hours). Keep at room temperature 8 days. Rehydrate for 10 minutes in 0.5 ml 50 mM MES prewarmed to 40°C.

6. Pellet one set and freeze at -20°C for determination of carbohydrate.

7. Dilute remaining two samples by adding 400 ul of MES. Freeze one sample by cooling to 4°C @ 4°C/min, 2°C @ 1°C/min, -20°C @ 0.5°C/min and holding at -20°C for 10 min. Thaw rapidly in water bath at 30°C.

8. Hold remaining sample at room temperature for control and plate during freezing of experimental cells.

9. Dilute cells in 50 mM MES. Plate (50 ul) in triplicate and compare frozen and dehydrated cells to control cells for viability count:
Dehydrated: 1:1000K--3; 1:100K--3; 1:10K--3; 1:100--3; 1:10--3; 1:1--3
Frozen, control: 1:1000K--3; 1:100K--3; 1:10K--3

Protocol for Figures 2 and 3
Examine growth of Δath1 strain in varying glucose concentrations.

1. Grow SEY6210 and dath1 in YPD (2%). Subculture and grow to O.D. = 3.0.

2. Inoculate YPD (5 ml) having various concentrations of glucose (0-40%). Start cultures at O.D. = 0.125.

3. Check O.D. after 24, 48 and 72 hours (50 ul in 2.45 ml).

4. Remove samples for ethanol, glucose and trehalose analysis. Remove 25 O.D.s of cells from each glucose concentration at the 48 hour time point and spin. Remove supernatant (1.0 ml) and freeze for analysis of glucose and ethanol levels. Wash pellet in 1X YNB and freeze for analysis of trehalose.

Protocol for figure 4
Examine effect of Δath1 mutation on survival following drying (growth in minimal medium).

1. Grow SEY6210 or SEY6210 dath1::URA3 in 100 ml YNBD medium (plus URA for both) to respiratory or stationary phase.

2. Spin 4 x 5 O.D.s [for trehalose assay: spin an additional 10 O.D.s] and wash in 1X YNB. Resuspend in 1.5 ml 1X YNB [for trehalose assay: wash twice, R/S in 100 ul and read O.D. of 5 ul. Freeze remainder].

3. Transfer 0.47 ml to three new microfuge tubes.

4. Read O.D.600 of remaining sample (25 ul in 1 ml).

5. Pellet two sets, remove supernatant and R/S in 120 ul of 1X YNB. Read O.D.600 of 5 ul. Transfer 100 ul to new tube, weigh and place in speed-vac. Lyophilize until constant weight (3-5 hours). Keep at room temperature for 33 days. Rehydrate one set in 1X YNB prewarmed to 40°C (0.47 ml) and plate in dilutions. Other set is a back-up.

6. Hold one set at room temperature for controls and plate during dehydration of experimental cells.

7. Dilute in 1X YNB. Plate (50 ul) in triplicate and compare dehydrated cells to control cells for viability count.

Strains and media: the *Escherichia coli* strains used in this study were MC1061 F^- *hsdR*$^-$ *hsdM*$^+$ *araD139* Δ*(araABOIC-leu)7679* Δ*lacX74 galU galK rpsL* (Casadaban and Cohen, 1980) and DH5a F^- ø80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17 supE44* l^- *thi-1 gyrA96 relA1*. The yeast strains used were SEY6210 *MAT*a *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9* and SEY6211 *MAT*a *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2Δ9*. Standard methods were used to construct yeast strain MDY3 *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9 Δath1::URA3*. Standard yeast (Sherman et al., 1979; Wickerham, 1946) and *E. coli* media (Miller, 1972) were used and supplemented as needed.

Reagents. YNB, Bacto Tryptone, Bacto Peptone, Bacto Yeast Extract and Bacto Agar were from Difco Laboratories (Detroit, Mich.). DNA restriction and modifying enzymes were from New England Biolabs, Inc. (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Mannheim, Germany). Hybond N^+ membranes for Southern and Northern (RNA) blots, [a-^{32}P]dCTP (3,000 Ci/mmol), and [^{35}S]dATPaS (>1,000 Ci/mmol) were from Amersham Buchler

(Braunschweig, Germany). Random priming materials and additional enzymes were from United States Biochemical Corp. (Cleveland, Ohio). Biochemical reagents were from Sigma (Deisenhofen, Germany).

Enzymatic overlay assay. Preparation and transformation of competent yeast cells by the lithium-acetate method was carried out as described by Ito (Ito et al., 1983). Yeast colonies transformed with DNA from a genomic plasmid library were replica-plated on YP (1% Bacto Yeast Extract, 2% Bacto Peptone) plates with 2% fructose as the carbohydrate source. After growth for 2 days at 30°C, 10 ml of an overlay-assay-mix that measures secreted ATH activity was poured onto each plate. The assay was performed as described previously (Kopp et al., 1993) with the following modifications: To prepare 100 ml of final volume of the overlay assay mix, 3.4 g of trehalose was dissolved in 80 ml of 200 mM citric acid, pH 4.5, 5 mM EDTA; 1g of agarose was added, and the mixture was melted in a microwave oven and then cooled to 50°C. Immediately before pouring the mixture onto the replica-plated colonies, 2 ml of *N*-ethylmaleimide (2.5 mg/ml), 985 units of horseradish peroxidase (EC 1.11.1.7), 800 units of glucose oxidase (EC 1.1.3.4), and 4.8 ml of *o*-dianisidine (10 mg/ml) were added. The overlay-assay mixture was incubated on the plates for 15 minutes at 24°C. Colonies with secreted acid trehalase activity developed a dark green color whereas the other transformants remained white.

Assays: liquid trehalase assays were performed as described previously (Kopp et al., 1993). Proteinase A was assayed according to Wiemken et al. (Wiemken et al., 1979), using denatured hemoglobin.

Amplification of Plasmid Library: a YEp24 genomic plasmid library (Carlson and Botstein, 1982) was kindly provided by D. Botstein (Stanford University). Amplification of the library was performed as described previously (Kopp et al., 1993).

Cloning, Sequencing and DNA Analysis of *ATH1*: genomic and plasmid DNA from *S. cerevisiae* and plasmid DNA from *E. coli* were prepared as described previously (Birnboim and Doly, 1979; Sherman et al., 1979). Standard procedures were followed for subcloning DNA fragments and for identifying

recombinant clones (Maniatis et al., 1982). After screening for secreted acid trehalase activity, plasmid DNA from the positive-reacting yeast transformant colonies was isolated (Sherman et al., 1979); plasmids containing 8.5 and 10-kbp inserts were recovered. To determine the nucleotide sequence, *SalI* and *EcoRI* restriction fragments from the 8.5-kbp insert were subcloned into the vectors pTZ18R and pTZ19R (Pharmacia, Freiburg, Germany) to construct plasmids pMATZ1 to pMATZ4. The nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977). The sequence containing the *ATH1* gene was determined on both the coding and noncoding strands.

The 0.3 kb *Eco*RI/*Sal*I fragment from pDAT1.9 was used as a probe in Southern (Southern, 1975) and Northern blot analyses. Radiolabeled DNA hybridization probes were prepared by the random priming method (Feinberg and Vogelstein, 1983). For Southern blot analysis, the genomic DNA was digested with *Eco*RI, separated on a 0.8% agarose gel, incubated in 0.25 M HCl, and blotted onto a Hybond N^+ membrane in 0.4 M NaOH. For Northern blot analysis, the RNA was prepared by the method of Chirgwin (Chirgwin et al., 1979). Following electrophoresis, the RNA was transferred to a Hybond N^+ membrane in 0.04 M NaOH.

Deletion of *ATH1*: Plasmid pMATZ1 contains two *Eco*RV sites, one in the 5′ non-coding region and the other within the open reading frame (Figure 4). This plasmid was restricted with *Eco*RV to remove a 2.4-kbp fragment encoding most of the open reading frame of *ATH1*. The 1.1-kbp *Hin*dIII fragment containing the *URA3* gene was isolated from plasmid YEp24, and the overhanging 5′-ends were filled in by treatment with the Klenow fragment of DNA polymerase I. The blunt-ended *URA3* fragment was cloned into pMATZ1 which had been digested with *Eco*RV to generate plasmid pMATZ1.1. The *Eco*RI fragment from plasmid pMATZ1.1 was isolated and used to transform yeast strain SEY6210 with approximately 10 μg of DNA. Ura^+ colonies were isolated and examined by Southern blotting to confirm the site of integration. Yeast strain MDY3 contained the *URA3* gene integrated at the chromosomal *ATH1* locus.

Identification of two ATH-secretin

gene encoding the vacuolar acid trehalase,

approaches. First, peptide sequences were obtained clone the structural

a protein fraction with high acid trehalase activity (Mitten wo independent

5 Degenerate oligonucleotides were synthesized based on the pe urification of

were used in a polymerase chain reaction with genomic DN zer, 1988).

(Destruelle et al., 1994). Cloning of the corresponding gene led to the ces and

of a novel yeast gene, *YGP1* (Destruelle et al., 1994). The *YGP1* gene codes late

a highly glycosylated, secreted protein with an unknown function. The *YGP1*

10 gene, however, is not the structural gene for acid trehalase. It appears that additional proteins may have been retained during the initial purification (Mittenbühler and Holzer, 1988) of acid trehalase.

In the second approach, we relied on the observation that overproduction of vacuolar proteins can lead to their expression at the cell surface (Rothman et al.,

15 1986; Stevens et al., 1986). Secreted proteins can then be identified by immunoblotting with a specific antibody or by their enzymatic activity. For the cloning of acid trehalase, we developed a specific enzymatic overlay assay that allowed the convenient screening of many transformants (see Materials and Methods). The activity of neutral trehalase was inhibited by the addition of 5 mM

20 EDTA and the acidic pH of 4.5, where the enzyme shows very little activity (App and Holzer, 1989). To identify putative ATH-encoding clones, yeast strain SEY6210 was transformed with plasmid DNA from a YEp24-based genomic library (Carlson and Botstein, 1982). Between 200 and 500 transformant colonies per plate were replica-plated on YNB-plates containing fructose as a carbon source

25 and assayed for secreted acid trehalase activity. In a screen of approximately 10,000 Ura^+ transformants, nine were positive for secreted ATH activity. Reintroducing the purified plasmids (pDAT1.1-pDAT1.9) into yeast resulted in the secretion of acid trehalase activity. Thus, pDAT1.1-pDAT1.9 carry DNA sequences that cause yeast cells to secrete a catalytically active portion of their acid

30 trehalase.

of the DNA fragment that confers an ATH secretion phenotype: mapping of the plasmid DNA isolated from the yeast cells revealed two plasmids with overlapping genomic inserts of 10 and 8.5-kbp. The plasmids were named pDAT1.8 and pDAT1.9, respectively. To define the limits of the DNA segment leading to acid trehalase secretion, various subclones of pDAT1.9 were constructed in plasmids YEp24 and pSEY8 (Emr et al., 1986), and yeast transformants carrying the subcloned plasmids were examined for secretion of ATH. None of the subclones showed secreted acid trehalase activity indicating that the functional gene sequence was larger than that contained on any of the subcloned fragments. Therefore, the entire nucleotide sequence of the 8.5-kbp insert was determined by sequence analysis. For that purpose, the *Eco*RI and *Sal*I-fragments from the genomic insert of pDAT1.9 were subcloned into the sequencing vectors pTZ18R and pTZ19R. Analysis of the sequence revealed two open reading frames from which one has been described previously as part of the *YMN1* gene (Thorsness et al., 1993). The second open reading frame (SEQUENCE ID NO:1), which showed no homology to sequences in the EMBL and GenBank nucleotide libraries, was 3,126 bp and is contained in the *Eco*RI fragment from pDAT1.9.7. The 5′ noncoding region contains two possible TATA boxes. The open reading frame encodes a 1,041 amino acid protein (SEQUENCE ID NO:2) with a predicted molecular mass of approximately 117,400 Da. The coding region contains 25 potential N-glycosylation sites. The gene was named *ATH1* (Accession Number: X84156 S. cerevisiae ATH1 gene), for yeast acid trehalase.

Characterization of *ATH1*: The amino acid sequence deduced from the *ATH1* gene was compared with those of proteins in the SWISS-Prot and PIR protein databases by use of the FASTA algorithm and the Wordsearch program of the University of Wisconsin Genetics Computer Group package (Devereux et al., 1984). This analysis did not reveal any homology to the five cloned trehalases from different organisms (Gutierrez et al., 1989; Kopp et al., 1993; Ruf et al., 1990; Su et al., 1993; Takiguchi et al., 1992) nor to any other protein in the libraries. Ath1p lacks a characteristic signal sequence at the amino terminus as expected for a soluble secretory pathway protein. In addition, there are no hydrophobic

domains that are likely to serve as internal signal sequences. The deduced amino acid sequence also does not reveal a consensus signal sequence cleavage site based on the rules of von Heijne (von Heijne, 1986); there are no positive S values indicating likely cleavage sites within the first N-terminal 300 amino acids. However, localization of Ath1p to the vacuole could occur by a mechanism independent of the secretory pathway (Klionsky et al., 1992). ATH has been characterized as a glycosylated protein that transits to the vacuole in a *sec*-dependent manner, however, suggesting movement through the secretory pathway (Londesborough and Varimo, 1984; Harris and Cotter, 1988; Mittenbühler and Holzer, 1988).

To confirm the requirement of the *ATH1* gene for acid trehalase activity, we carried out a one step gene transplacement (Rothstein, 1983). The *ATH1* gene was disrupted at the chromosomal locus to generate the mutant yeast strain MDY3. The mutant strain has no detectable acid trehalase activity as determined using the overlay assay or liquid assays with crude cell extracts (Table 1).

Table 1. Enzymatic activities of vacuolar and cytosolic proteins in wild type strains and a strain overexpressing the *ATH1* gene.

Strain	ATH activity (mU/mg)	NTH activity (mU/mg)	Alkaline phosphatase activity (mU/mg)	Glucose-6-phosphate Proteinase A dehydrogenase activity (mU/mg)
SEY6210	8.5	19.5	100	8.6160
SEY6210/YEp24	6.5	20.1	60	7.5175
SEY6210/pDAT1.9	67.0	26.1	70	7.6200

These results indicate that the *ATH1* gene product is required for ATH activity but do not demonstrate whether *ATH1* is the structural gene for acid trehalase or encodes a regulatory protein.

Northern blot analysis revealed that *ATH1* is expressed in stationary phase cells while no expression could be detected in logarithmically growing yeast cells. The expression pattern corresponds with the activity profile of acid trehalase. To further investigate if *ATH1* is the structural gene for acid trehalase or a putative regulator, activities of different vacuolar and cytosolic enzymes were measured in the acid trehalase-overproducing strain, the mutant strain MDY3 and in a wild type strain. As shown in Table 1, cells containing pDAT1.9 exhibit about an 8-10 fold higher level of acid trehalase activity than the same strain carrying the parent 2μ plasmid YEp24. Of the enzyme activities examined, only acid trehalase is dramatically increased in cells containing pDAT1.9; the activities of other vacuolar proteins (alkaline phosphatase and proteinase A) and a cytosolic protein (glucose-6-phosphate dehydrogenase) are not elevated. The activity of neutral trehalase is slightly increased upon overproduction of *ATH1*. In the Δ*ath1* strain, however, ATH activity is completely eliminated while there is no effect on NTH activity. The enzymatic activities of acid and neutral trehalases in the Δ*ath1* strain are at the same levels as are seen in an acid trehalase mutant generated by random mutagenesis with ethyl methane sulfonate (Destruelle, 1993); the mutation completely eliminates ATH activity while having no effect on the activity of NTH. The plasmid pDAT1.9 complements the mutagen-induced defect but is not able to complement a mutant lacking neutral trehalase activity (Destruelle, 1993).

References

App and Holzer (1989) *J. Biol. Chem.* **264**:17583-17588; Arguelles (1994) *FEBS Lett.* **350**:266-270; Birnboim and Doly (1979) *Nucl. Acids Res.* **7**:1513-1523; Carlson and Botstein (1982) *Cell* **28**:145-154; Casadaban and Cohen (1980) *J. Mol. Biol.* **138**:179-207; Chirgwin et al., (1979) *Biochemistry* **18**:5294-5299; Destruelle (1993) Ph.D. thesis, Faculty of Biology, University of Freiburg, Germany; Destruelle et al., (1994) *Mol. Cell Biol.* **14**:2740-2754; Devereux et al., (1984)

Nucl. Acid. Res. **12**:387-395; Elbein (1974) *Adv. Carbohydr. Chem. Biochem.* **30**:227-256; Emr et al., (1986) *J. Cell Biol.* **102**:523-533; Feinberg et al., (1983) *Anal. Biochem.* **132**:6-13; Gelinas et al., (1989) *Appl. Environ. Microbiol.* **55**:2453-2459; Guarente (1984) *Cell* **36**:285-315; Gutierrez et al., (1989) *Mol. Gen. Genet.* **217**:347-354; Harris and Cotter (1987) *Current Microbiol.* **15**:247-249; Harris and Cotter (1988) *Can. J. Microbiol.* **34**:835-838; Hino et al., (1990) *Appl. Environ. Microbiol.* **56**:1386-1391; Ito et al., (1983) *J. Bacteriol.* **153**:163-168; Klionsky et al., (1992) *J. Cell Biol.* **119**, 287-299; Kopp et al., (1993) *J. Biol. Chem.* **268**:4766-4774; Londesborough and Varimo (1984) *Biochem. J.* **219**:511-518; Maniatis et al., (1982) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press, New York; Mansure et al., (1994) *Biochem. Biophys. Acta* **1191**:309-316; Miller (1972) *Experiments in molecular genetics* Cold Spring Harbor Laboratory Press, New York; Mittenbühler and Holzer (1988) *J. Biol. Chem.* **263**:8537-8543; Mittenbühler and Holzer (1991) *Arch. Microbiol.* **155**:217-220; Nwaka et al. (1994) *FEBS Lett.* **344**:225-228; Oda et al., (1986) *Appl. Environ. Microbiol.* **52**:941-943; Rine (1991) *Methods Enzymol.* **194**:239-251; Rothman (1986) *Proc. Natl. Acad. Sci. USA* **83**:3248-3252; Rothstein (1983) *Methods Enzymol.* **101**:202-211; Ruf et al., (1990) *J. Biol. Chem.* **265**:15034-15039; Sanger et al., (1977) *Proc. Natl. Acad. Sci. USA* **76**:5463-5467; Sherman et al., (1979) *Methods in Yeast Genetics: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York; Southern (1975) *J. Mol. Biol.* **98**:503-517; Stevens et al., (1986) *J. Cell Biol.* **102**:1551-1557; Su et al., (1993) *Biochim. Biophys. Acta* **1173**:217-224; Takiguchi et al., (1992) *Biochem. J.* **288**:19-22; Thevelein (1984) *Microbiol. Rev.* **48**:42-59; Thorsness et al., (1993) *Mol. Cell Biol.* **13**:5418-5426; von Heijne (1986) *Nucl. Acids Res.* **14**:4683-4690; Vuorio et al., (1993) *Eur. J. Biochem.* **216**:849-861; Wickerham (1946) *J. Bacteriol.* **52**:293-301; Wiemken (1990) *Antonie Van Leeuwenhoek* **58**:209-217; Wiemken et al., (1979) *Arch. Microbiol.* **123**:23-35; Wiggers (1832) *Ann. Pharm (Pozna)* **1**:129-182; Winkler et al., (1991) *FEBS Lett.* **291**:269-272.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application

were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Regents of the University of California

(ii) TITLE OF INVENTION: STRESS TOLERANT YEAST MUTANTS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Berliner, Robert
(B) REGISTRATION NUMBER: 20,121
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(B) TELEFAX: (213) 977-1003
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3876 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 579..3701

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCGTTATTG AATAATAATA ACAAAATTTC TCCACTTTTC ACCATATTAC CACTCCCTTT 60

TTTTCAAGTA AAAAAAAAAA AAAAAAGAAT CTTATTGCTC CTTAAAAAAG GATTCATCAG 120

TCCTTTATGA GGCCTGCTAG TATCACCTAA TATTGCATCT GTTTTTACCG AAATTTCCTC 180

ATTCCAATAA TGAAAAGAAT AAGATCGCTT TGGTTCAATG CGGAGGCTTC TTATTCAAAC 240

CTTAACAATT CTCCTAGTTT GAGGAACAAA AATAGTACCG GTAATAACTC TCGCTCTAAA 300

AATTATCGTT CTTTCTCAAG GTTTGACTTG ATCAACTCTA TACTTTTACT GATGATGCTA 360

TTTTTATTAG CTATCTTCGT CACTGCATTA TATTTAACAA AGCGTTCCAG GCTTACATAC 420

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TCACATGCCT CGAGGGCTGC CCTATTTAAC CTCTGGGTGT GATATCGCCG TCATTGGGAA      480

ATCATACGTT GAACTACGAT CCAGAAGCAA GGGAATCTTC TAAAAAACTT TATGAACTCC      540

TTTCTGATTT CAACACGGCA TATTATGATG ATGAGAAC ATG ATT TTG GGA AGT           593
                                             Met Ile Leu Gly Ser
                                               1               5

AAC TTG TTC TCA AAG AAT ACA TAC TCG AGA CAA CCA TAT GTT GCT AAC         641
Asn Leu Phe Ser Lys Asn Thr Tyr Ser Arg Gln Pro Tyr Val Ala Asn
                10                  15                  20

GGT TAT ATA GGT AGT CGT ATT CCC AAT ATT GGG TTC GGC TAT GCC TTA         689
Gly Tyr Ile Gly Ser Arg Ile Pro Asn Ile Gly Phe Gly Tyr Ala Leu
            25                  30                  35

GAC ACC CTG AAT TTT TAC ACA GAC GCA CCA GGC GCT TTG AAT AAC GGT         737
Asp Thr Leu Asn Phe Tyr Thr Asp Ala Pro Gly Ala Leu Asn Asn Gly
        40                  45                  50

TGG CCC TTA AGA AAT CAT AGA TTT GCC GGT GCG TTT GTA TCG GAC TTT         785
Trp Pro Leu Arg Asn His Arg Phe Ala Gly Ala Phe Val Ser Asp Phe
    55                  60                  65

TAT TGT CTA CAA CCA AAA CTA AAT TCA ACA AAC TTC CCA GAA TTG GAT         833
Tyr Cys Leu Gln Pro Lys Leu Asn Ser Thr Asn Phe Pro Glu Leu Asp
70                  75                  80                  85

GAT GTA GGA TAT TCC ACT GTC ATT TCA TCT ATT CCA CAA TGG ACC AAT         881
Asp Val Gly Tyr Ser Thr Val Ile Ser Ser Ile Pro Gln Trp Thr Asn
                90                  95                  100

CTA CAG TTC TCA TTA GTG AAT GAT TCT AAG TGG TTC AAT CCA CAA AAT         929
Leu Gln Phe Ser Leu Val Asn Asp Ser Lys Trp Phe Asn Pro Gln Asn
            105                 110                 115

GTT ACG TTG GAT GAC GTA ACT AAT TAT AGC CAA AAC TTA TCA ATG AAG         977
Val Thr Leu Asp Asp Val Thr Asn Tyr Ser Gln Asn Leu Ser Met Lys
        120                 125                 130

GAT GGT ATC GTA ACT ACG GAG TTA GAT TGG CTA AAC AGT CAA ATA CAT        1025
Asp Gly Ile Val Thr Thr Glu Leu Asp Trp Leu Asn Ser Gln Ile His
    135                 140                 145

GTT AAA AGT GAA ATC TGG GCA CAT CGG CAC ATT CAT CCA CTG GGA GTG        1073
Val Lys Ser Glu Ile Trp Ala His Arg His Ile His Pro Leu Gly Val
150                 155                 160                 165

GTT TCT TTG GAA ATT TCC CTG AAT ACG GAC CAT TTA CCT TCG GAT TTT        1121
Val Ser Leu Glu Ile Ser Leu Asn Thr Asp His Leu Pro Ser Asp Phe
                170                 175                 180

GAT TCA TTA GAT GTT AAT ATA TGG GAT ATA CTT GAT TTC AAC ACA TCA        1169
Asp Ser Leu Asp Val Asn Ile Trp Asp Ile Leu Asp Phe Asn Thr Ser
            185                 190                 195

CAT AGG ACT GTT CTA CAT AGC ACG GGA ACA GAC GAA AAA AAT AAT GCG        1217
His Arg Thr Val Leu His Ser Thr Gly Thr Asp Glu Lys Asn Asn Ala
        200                 205                 210

GTT TTC ATG ATT GTT CAG CCA GAT AAC GTT CCA TCT TCT AAT TGC GCT        1265
Val Phe Met Ile Val Gln Pro Asp Asn Val Pro Ser Ser Asn Cys Ala
    215                 220                 225

ATT TAC TCA ACG TGT ACT GTA AAG TAT GAA AAT TCC ACC AAT CCA ATA        1313
Ile Tyr Ser Thr Cys Thr Val Lys Tyr Glu Asn Ser Thr Asn Pro Ile
230                 235                 240                 245

AAT TCT AGT GAA TCT TTT GAA GAA AAA GAT GTT TCT TCT AAT ATT TAT        1361
Asn Ser Ser Glu Ser Phe Glu Glu Lys Asp Val Ser Ser Asn Ile Tyr
                250                 255                 260
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AAT GTT ATT TTG AGA GAG GAC CAA CCC AAG ATA ATC GTT CAT AAG TAT     1409
Asn Val Ile Leu Arg Glu Asp Gln Pro Lys Ile Ile Val His Lys Tyr
            265                 270                 275

GTT GGT ATT ATG TCT ACT GAG TTC AAT AAG AAC AAA GAA CAA CAA GAC     1457
Val Gly Ile Met Ser Thr Glu Phe Asn Lys Asn Lys Glu Gln Gln Asp
        280                 285                 290

AAT ACT AAT ATT GGT TTG GCC AAA ATG ATA GCT CTA AAC AGT AAA GGC     1505
Asn Thr Asn Ile Gly Leu Ala Lys Met Ile Ala Leu Asn Ser Lys Gly
    295                 300                 305

AAT TAC GAG AAG CTT CTG TCA AGT CAC AAA CGT GCG TGG TAT GAC CTT     1553
Asn Tyr Glu Lys Leu Leu Ser Ser His Lys Arg Ala Trp Tyr Asp Leu
310                 315                 320                 325

TAC AAC GAC GCC TTC ATT GAA ATT CCT TCT GAC AGT CTT TTA GAA ATG     1601
Tyr Asn Asp Ala Phe Ile Glu Ile Pro Ser Asp Ser Leu Leu Glu Met
                330                 335                 340

ACA GCA AGA TCG TCC CTA TTC CAT TTA CTA GCA AAT ACA AGA GAT TAC     1649
Thr Ala Arg Ser Ser Leu Phe His Leu Leu Ala Asn Thr Arg Asp Tyr
            345                 350                 355

AAT GTC TCG AGC GAT AGG GGT CTT CCC GTG GGA GTT TCT GGT TTG TCA     1697
Asn Val Ser Ser Asp Arg Gly Leu Pro Val Gly Val Ser Gly Leu Ser
        360                 365                 370

TCA GAT TCC TAT GGT GGT ATG GTG TTC TGG GAC GCA GAT ATA TGG ATG     1745
Ser Asp Ser Tyr Gly Gly Met Val Phe Trp Asp Ala Asp Ile Trp Met
    375                 380                 385

GAA CCT GCC CTA TTG CCT TTC TTC CCA AAT GTG GCT CAA AAT ATG AAT     1793
Glu Pro Ala Leu Leu Pro Phe Phe Pro Asn Val Ala Gln Asn Met Asn
390                 395                 400                 405

AAT TAC AGA AAT GCT ACA CAT TCG CAG GCA AAG TTA AAT GCA GAG AAA     1841
Asn Tyr Arg Asn Ala Thr His Ser Gln Ala Lys Leu Asn Ala Glu Lys
                410                 415                 420

TAT GGA TAC CCC GGA GCA ATA TAC CCC TGG ACA TCT GGT AAG TAC GCT     1889
Tyr Gly Tyr Pro Gly Ala Ile Tyr Pro Trp Thr Ser Gly Lys Tyr Ala
            425                 430                 435

AAT TGT ACT TCT ACG GGA CCT TGT GTC GAT TAC GAA TAC CAT ATT AAC     1937
Asn Cys Thr Ser Thr Gly Pro Cys Val Asp Tyr Glu Tyr His Ile Asn
        440                 445                 450

GTT GAT GTC GCT ATG GCC TCC TTT TCC ATA TAC TTG AAT GGA CAC GAA     1985
Val Asp Val Ala Met Ala Ser Phe Ser Ile Tyr Leu Asn Gly His Glu
    455                 460                 465

GGG ATT GAT GAC GAG TAT CTG AGA TAT ACT ACA TGG CCA ATT ATC AAA     2033
Gly Ile Asp Asp Glu Tyr Leu Arg Tyr Thr Thr Trp Pro Ile Ile Lys
470                 475                 480                 485

AAC GCA GCC CAA TTT TTT ACT GCT TAT GTT AAG TAC AAT TCT TCC CTA     2081
Asn Ala Ala Gln Phe Phe Thr Ala Tyr Val Lys Tyr Asn Ser Ser Leu
                490                 495                 500

GGA TTG TAT GAA ACA TAT AAT TTG ACA GAT CCC GAC GAG TTT GCT AAT     2129
Gly Leu Tyr Glu Thr Tyr Asn Leu Thr Asp Pro Asp Glu Phe Ala Asn
            505                 510                 515

CAC ATC AAT AAC GGG GCT TTC ACG AAT GCT GGC ATT AAA ACA CTT CTA     2177
His Ile Asn Asn Gly Ala Phe Thr Asn Ala Gly Ile Lys Thr Leu Leu
        520                 525                 530

AAG TGG GCA ACA GAC ATT GGC AAT CAT CTC GGC GAG GTC GTT GAC CCC     2225
Lys Trp Ala Thr Asp Ile Gly Asn His Leu Gly Glu Val Val Asp Pro
    535                 540                 545
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AAA TGG AGT GAA ATT TCC AAA GAT ATT TAT ATC CCT AGA TCC TCA TCT     2273
Lys Trp Ser Glu Ile Ser Lys Asp Ile Tyr Ile Pro Arg Ser Ser Ser
550                 555                 560                 565

AAC ATC ACT TTG GAA TAT TCT GGT ATG AAT AGC TCA GTG GAG ATT AAA     2321
Asn Ile Thr Leu Glu Tyr Ser Gly Met Asn Ser Ser Val Glu Ile Lys
                570                 575                 580

CAG GCG GAT GTG ACT TTA ATG GTT TAC CCA CTT GGA TAT ATC AAT GAT     2369
Gln Ala Asp Val Thr Leu Met Val Tyr Pro Leu Gly Tyr Ile Asn Asp
            585                 590                 595

GAA TCC ATA TTG AAC AAT GCA ATT AAA GAT CTT TAT TAT TAT TCA GAA     2417
Glu Ser Ile Leu Asn Asn Ala Ile Lys Asp Leu Tyr Tyr Tyr Ser Glu
        600                 605                 610

AGA CAG TCT GCG TCT GGG CCT GCA ATG ACA TAT CCG GTT TTT GTG GCC     2465
Arg Gln Ser Ala Ser Gly Pro Ala Met Thr Tyr Pro Val Phe Val Ala
    615                 620                 625

GCA GCT GCT GGT CTG CTG AAT CAC GGC TCT TCT TCT CAA AGT TAC TTA     2513
Ala Ala Ala Gly Leu Leu Asn His Gly Ser Ser Ser Gln Ser Tyr Leu
630                 635                 640                 645

TAT AAA TCG GTT CTT CCA TAC TTA CGG GCT CCT TTC GCT CAA TTT AGT     2561
Tyr Lys Ser Val Leu Pro Tyr Leu Arg Ala Pro Phe Ala Gln Phe Ser
                650                 655                 660

GAG CAA TCA GAC GAC AAC TTT TTA ACA AAC GGA TTA ACC CAG CCA GCA     2609
Glu Gln Ser Asp Asp Asn Phe Leu Thr Asn Gly Leu Thr Gln Pro Ala
            665                 670                 675

TTC CCC TTT TTA ACA GCT AAC GGT GGA TTT CTA CAG AGC ATT CTG TTT     2657
Phe Pro Phe Leu Thr Ala Asn Gly Gly Phe Leu Gln Ser Ile Leu Phe
        680                 685                 690

GGG TTA ACA GGA ATC CGA TAT TCT TAT GAG GTT GAT CCA GAT ACT AAA     2705
Gly Leu Thr Gly Ile Arg Tyr Ser Tyr Glu Val Asp Pro Asp Thr Lys
    695                 700                 705

AAA ATT AAC CGT TTG TTA AGG TTC AAT CCA ATA GAA CTA CCG TTG CTC     2753
Lys Ile Asn Arg Leu Leu Arg Phe Asn Pro Ile Glu Leu Pro Leu Leu
710                 715                 720                 725

CCT GGT GGT ATC GCT ATT AGA AAC TTC AAA TAT ATG AAC CCA GTT TTA     2801
Pro Gly Gly Ile Ala Ile Arg Asn Phe Lys Tyr Met Asn Pro Val Leu
                730                 735                 740

GAT ATA ATA ATT GAC GAC CAC AAT GGT ACG ATT GTT CAT AAA TCA GGA     2849
Asp Ile Ile Ile Asp Asp His Asn Gly Thr Ile Val His Lys Ser Gly
            745                 750                 755

GAT GTT CCT ATT CAT ATA AAG ATA CCA AAC AGA TCT CTA ATA CAT GAC     2897
Asp Val Pro Ile His Ile Lys Ile Pro Asn Arg Ser Leu Ile His Asp
        760                 765                 770

CAG GAT ATC AAC TTC TAT AAT GGT TCC GAA AAC GAA AGA AAA CCA AAT     2945
Gln Asp Ile Asn Phe Tyr Asn Gly Ser Glu Asn Glu Arg Lys Pro Asn
    775                 780                 785

CTA GAG CGT AGA GAC GTC GAC CGT GTT GGT GAT CCA ATG AGG ATG GAT     2993
Leu Glu Arg Arg Asp Val Asp Arg Val Gly Asp Pro Met Arg Met Asp
790                 795                 800                 805

AGG TAT GGT ACC TAT TAT CTT TTA AAA CCG AAA CAA GAG CTT ACA GTC     3041
Arg Tyr Gly Thr Tyr Tyr Leu Leu Lys Pro Lys Gln Glu Leu Thr Val
                810                 815                 820

CAA CTG TTC AAG CCT GGC TTA AAC GCA AGA AAC AAC ATA GCG GAA AAT     3089
Gln Leu Phe Lys Pro Gly Leu Asn Ala Arg Asn Asn Ile Ala Glu Asn
            825                 830                 835
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AAG CAA ATA ACA AAC TTG ACG GCC GGC GTT CCT GGT GAC GTT GCA TTC     3137
Lys Gln Ile Thr Asn Leu Thr Ala Gly Val Pro Gly Asp Val Ala Phe
        840                 845                 850

TCT GCT CTA GAT GGG AAT AAT TAC ACG CAT TGG CAA CCC TTA GAC AAA     3185
Ser Ala Leu Asp Gly Asn Asn Tyr Thr His Trp Gln Pro Leu Asp Lys
    855                 860                 865

ATT CAC CGT GCG AAG CTA TTG ATT GAT TTA GGT GAA TAC AAC GAG AAA     3233
Ile His Arg Ala Lys Leu Leu Ile Asp Leu Gly Glu Tyr Asn Glu Lys
870                 875                 880                 885

GAG ATT ACC AAG GGA ATG ATT CTT TGG GGG CAG AGG CCC GCA AAA AAC     3281
Glu Ile Thr Lys Gly Met Ile Leu Trp Gly Gln Arg Pro Ala Lys Asn
            890                 895                 900

ATT TCC ATA TCT ATT TTG CCT CAT TCT GAA AAA GTC GAA AAT TTA TTT     3329
Ile Ser Ile Ser Ile Leu Pro His Ser Glu Lys Val Glu Asn Leu Phe
        905                 910                 915

GCG AAC GTG ACA GAA ATT ATG CAA AAT TCG GGA AAT GAT CAA CTT CTT     3377
Ala Asn Val Thr Glu Ile Met Gln Asn Ser Gly Asn Asp Gln Leu Leu
        920                 925                 930

AAT GAA ACC ATT GGT CAG CTT TTA GAT AAT GCC GGA ATT CCT GTC GAG     3425
Asn Glu Thr Ile Gly Gln Leu Leu Asp Asn Ala Gly Ile Pro Val Glu
    935                 940                 945

AAC GTT ATT GAT TTT GAT GGC ATA GAA CAA GAG GAT GAT GAA TCT TTG     3473
Asn Val Ile Asp Phe Asp Gly Ile Glu Gln Glu Asp Asp Glu Ser Leu
950                 955                 960                 965

GAT GAT GTG CAA GCC TTA TTG CAC TGG AAG AAG GAA GAC TTA GCC AAG     3521
Asp Asp Val Gln Ala Leu Leu His Trp Lys Lys Glu Asp Leu Ala Lys
                970                 975                 980

CTA ATT GAC CAA ATA CCC AGA CTT AAC TTT CTA AAA AGA AAA TTT GTG     3569
Leu Ile Asp Gln Ile Pro Arg Leu Asn Phe Leu Lys Arg Lys Phe Val
            985                 990                 995

AAA ATT CTG GAT AAC GTG CCA GTG AGC CCA AGT GAG CCA TAC TAC GAA     3617
Lys Ile Leu Asp Asn Val Prc Val Ser Pro Ser Glu Pro Tyr Tyr Glu
        1000                1005                1010

GCA AGT CGC AAC CAG TCG TTA ATC GAG ATA TTA CCC AGT AAT AGA ACG     3665
Ala Ser Arg Asn Gln Ser Leu Ile Glu Ile Leu Pro Ser Asn Arg Thr
    1015                1020                1025

ACT TTC ACT ATT GAT TAT GAT AAA TTT GCA GGT GGG TGATAAAGGG          3711
Thr Phe Thr Ile Asp Tyr Asp Lys Phe Ala Gly Gly
1030                1035                1040

AACACAGATT GGAGGAAAAC AAGATACATA GTTGTTTCGT ACAAGGAGTG TATGATGATT   3771

ATATGATGAT AACAAAGGAG CTACAATCAA GGAAATTGTT CTCAATGATT AAATGAAATG   3831

ATGCATATTA GTAGCGCTTT TTTTAATATT ATAAGTTTGG ATAAA                   3876
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1041 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ile Leu Gly Ser Asn Leu Phe Ser Lys Asn Thr Tyr Ser Arg Gln
 1               5                  10                  15
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Pro Tyr Val Ala Asn Gly Tyr Ile Gly Ser Arg Ile Pro Asn Ile Gly
            20                  25                  30

Phe Gly Tyr Ala Leu Asp Thr Leu Asn Phe Tyr Thr Asp Ala Pro Gly
        35                  40                  45

Ala Leu Asn Asn Gly Trp Pro Leu Arg Asn His Arg Phe Ala Gly Ala
    50                  55                  60

Phe Val Ser Asp Phe Tyr Cys Leu Gln Pro Lys Leu Asn Ser Thr Asn
65                  70                  75                  80

Phe Pro Glu Leu Asp Asp Val Gly Tyr Ser Thr Val Ile Ser Ser Ile
                85                  90                  95

Pro Gln Trp Thr Asn Leu Gln Phe Ser Leu Val Asn Asp Ser Lys Trp
            100                 105                 110

Phe Asn Pro Gln Asn Val Thr Leu Asp Asp Val Thr Asn Tyr Ser Gln
        115                 120                 125

Asn Leu Ser Met Lys Asp Gly Ile Val Thr Thr Glu Leu Asp Trp Leu
    130                 135                 140

Asn Ser Gln Ile His Val Lys Ser Glu Ile Trp Ala His Arg His Ile
145                 150                 155                 160

His Pro Leu Gly Val Val Ser Leu Glu Ile Ser Leu Asn Thr Asp His
                165                 170                 175

Leu Pro Ser Asp Phe Asp Ser Leu Asp Val Asn Ile Trp Asp Ile Leu
            180                 185                 190

Asp Phe Asn Thr Ser His Arg Thr Val Leu His Ser Thr Gly Thr Asp
        195                 200                 205

Glu Lys Asn Asn Ala Val Phe Met Ile Val Gln Pro Asp Asn Val Pro
    210                 215                 220

Ser Ser Asn Cys Ala Ile Tyr Ser Thr Cys Thr Val Lys Tyr Glu Asn
225                 230                 235                 240

Ser Thr Asn Pro Ile Asn Ser Ser Glu Ser Phe Glu Glu Lys Asp Val
                245                 250                 255

Ser Ser Asn Ile Tyr Asn Val Ile Leu Arg Glu Asp Gln Pro Lys Ile
            260                 265                 270

Ile Val His Lys Tyr Val Gly Ile Met Ser Thr Glu Phe Asn Lys Asn
        275                 280                 285

Lys Glu Gln Gln Asp Asn Thr Asn Ile Gly Leu Ala Lys Met Ile Ala
    290                 295                 300

Leu Asn Ser Lys Gly Asn Tyr Glu Lys Leu Leu Ser Ser His Lys Arg
305                 310                 315                 320

Ala Trp Tyr Asp Leu Tyr Asn Asp Ala Phe Ile Glu Ile Pro Ser Asp
                325                 330                 335

Ser Leu Leu Glu Met Thr Ala Arg Ser Ser Leu Phe His Leu Leu Ala
            340                 345                 350

Asn Thr Arg Asp Tyr Asn Val Ser Ser Asp Arg Gly Leu Pro Val Gly
        355                 360                 365

Val Ser Gly Leu Ser Ser Asp Ser Tyr Gly Gly Met Val Phe Trp Asp
    370                 375                 380

Ala Asp Ile Trp Met Glu Pro Ala Leu Leu Pro Phe Phe Pro Asn Val
385                 390                 395                 400
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Ala Gln Asn Met Asn Asn Tyr Arg Asn Ala Thr His Ser Gln Ala Lys
                405                 410                 415

Leu Asn Ala Glu Lys Tyr Gly Tyr Pro Gly Ala Ile Tyr Pro Trp Thr
            420                 425                 430

Ser Gly Lys Tyr Ala Asn Cys Thr Ser Thr Gly Pro Cys Val Asp Tyr
        435                 440                 445

Glu Tyr His Ile Asn Val Asp Val Ala Met Ala Ser Phe Ser Ile Tyr
    450                 455                 460

Leu Asn Gly His Glu Gly Ile Asp Asp Glu Tyr Leu Arg Tyr Thr Thr
465                 470                 475                 480

Trp Pro Ile Ile Lys Asn Ala Ala Gln Phe Phe Thr Ala Tyr Val Lys
                485                 490                 495

Tyr Asn Ser Ser Leu Gly Leu Tyr Glu Thr Tyr Asn Leu Thr Asp Pro
            500                 505                 510

Asp Glu Phe Ala Asn His Ile Asn Asn Gly Ala Phe Thr Asn Ala Gly
        515                 520                 525

Ile Lys Thr Leu Leu Lys Trp Ala Thr Asp Ile Gly Asn His Leu Gly
    530                 535                 540

Glu Val Val Asp Pro Lys Trp Ser Glu Ile Ser Lys Asp Ile Tyr Ile
545                 550                 555                 560

Pro Arg Ser Ser Ser Asn Ile Thr Leu Glu Tyr Ser Gly Met Asn Ser
                565                 570                 575

Ser Val Glu Ile Lys Gln Ala Asp Val Thr Leu Met Val Tyr Pro Leu
            580                 585                 590

Gly Tyr Ile Asn Asp Glu Ser Ile Leu Asn Asn Ala Ile Lys Asp Leu
        595                 600                 605

Tyr Tyr Tyr Ser Glu Arg Gln Ser Ala Ser Gly Pro Ala Met Thr Tyr
    610                 615                 620

Pro Val Phe Val Ala Ala Ala Ala Gly Leu Leu Asn His Gly Ser Ser
625                 630                 635                 640

Ser Gln Ser Tyr Leu Tyr Lys Ser Val Leu Pro Tyr Leu Arg Ala Pro
                645                 650                 655

Phe Ala Gln Phe Ser Glu Gln Ser Asp Asp Asn Phe Leu Thr Asn Gly
            660                 665                 670

Leu Thr Gln Pro Ala Phe Pro Phe Leu Thr Ala Asn Gly Gly Phe Leu
        675                 680                 685

Gln Ser Ile Leu Phe Gly Leu Thr Gly Ile Arg Tyr Ser Tyr Glu Val
    690                 695                 700

Asp Pro Asp Thr Lys Lys Ile Asn Arg Leu Leu Arg Phe Asn Pro Ile
705                 710                 715                 720

Glu Leu Pro Leu Leu Pro Gly Gly Ile Ala Ile Arg Asn Phe Lys Tyr
                725                 730                 735

Met Asn Pro Val Leu Asp Ile Ile Ile Asp Asp His Asn Gly Thr Ile
            740                 745                 750

Val His Lys Ser Gly Asp Val Pro Ile His Ile Lys Ile Pro Asn Arg
        755                 760                 765

Ser Leu Ile His Asp Gln Asp Ile Asn Phe Tyr Asn Gly Ser Glu Asn
    770                 775                 780
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Glu Arg Lys Pro Asn Leu Glu Arg Arg Asp Val Asp Arg Val Gly Asp
785                 790                 795                 800

Pro Met Arg Met Asp Arg Tyr Gly Thr Tyr Tyr Leu Leu Lys Pro Lys
                805                 810                 815

Gln Glu Leu Thr Val Gln Leu Phe Lys Pro Gly Leu Asn Ala Arg Asn
            820                 825                 830

Asn Ile Ala Glu Asn Lys Gln Ile Thr Asn Leu Thr Ala Gly Val Pro
        835                 840                 845

Gly Asp Val Ala Phe Ser Ala Leu Asp Gly Asn Asn Tyr Thr His Trp
    850                 855                 860

Gln Pro Leu Asp Lys Ile His Arg Ala Lys Leu Leu Ile Asp Leu Gly
865                 870                 875                 880

Glu Tyr Asn Glu Lys Glu Ile Thr Lys Gly Met Ile Leu Trp Gly Gln
                885                 890                 895

Arg Pro Ala Lys Asn Ile Ser Ile Ser Ile Leu Pro His Ser Glu Lys
            900                 905                 910

Val Glu Asn Leu Phe Ala Asn Val Thr Glu Ile Met Gln Asn Ser Gly
        915                 920                 925

Asn Asp Gln Leu Leu Asn Glu Thr Ile Gly Gln Leu Leu Asp Asn Ala
    930                 935                 940

Gly Ile Pro Val Glu Asn Val Ile Asp Phe Asp Gly Ile Glu Gln Glu
945                 950                 955                 960

Asp Asp Glu Ser Leu Asp Asp Val Gln Ala Leu Leu His Trp Lys Lys
                965                 970                 975

Glu Asp Leu Ala Lys Leu Ile Asp Gln Ile Pro Arg Leu Asn Phe Leu
            980                 985                 990

Lys Arg Lys Phe Val Lys Ile Leu Asp Asn Val Pro Val Ser Pro Ser
        995                 1000                1005

Glu Pro Tyr Tyr Glu Ala Ser Arg Asn Gln Ser Leu Ile Glu Ile Leu
    1010                1015                1020

Pro Ser Asn Arg Thr Thr Phe Thr Ile Asp Tyr Asp Lys Phe Ala Gly
1025                1030                1035                1040

Gly
```

WHAT IS CLAIMED IS:

1. An isolated yeast mutant deficient in the expression of functional Ath1p gene product.

2. An isolated yeast according to claim 1, wherein said mutant or an ancestor of said mutant was generated by genetically engineering a yeast cell to create a mutation in an Ath1p allele of said yeast cell.

3. An isolated yeast according to claim 1, wherein said mutant expresses less than 10% of that expressed by the corresponding wild-type yeast.

4. An isolated yeast mutant according to claim 1, as deposited in the ATCC deposit # _____.

5. An isolated nucleic acid comprising ATH1 (SEQUENCE ID NO:1) or fragment thereof capable of hybridizing under stringent conditions with ATH1.

6. An isolated nucleic acid according to claim 5 comprising in 5′ - 3′ orientation, a first ATH1 fragment thereof capable of hybridizing under stringent conditions with ATH1, an intervening sequence, and a second different ATH1 fragment thereof capable of hybridizing under stringent conditions with ATH1

7. A method for producing a yeast mutant with improved survival ability under stress conditions, said method comprising steps:

subjecting a population of yeast to stress conditions;

detecting in said population a yeast mutant deficient in the expression of functional Ath1p gene product;

isolating said yeast mutant; and,

growing said yeast mutant to obtain yeast with improved survival ability under stress conditions.

8. A method according to claim 7, said detecting step comprises contacting said population with an Ath1p gene product-specific reagent.

9. A method according to claim 7, said detecting step comprising contacting said population with an isolated nucleic acid comprising ATH1 (SEQUENCE ID NO:1) or fragment thereof capable of hybridizing under stringent conditions with ATH1.

FIG. 1

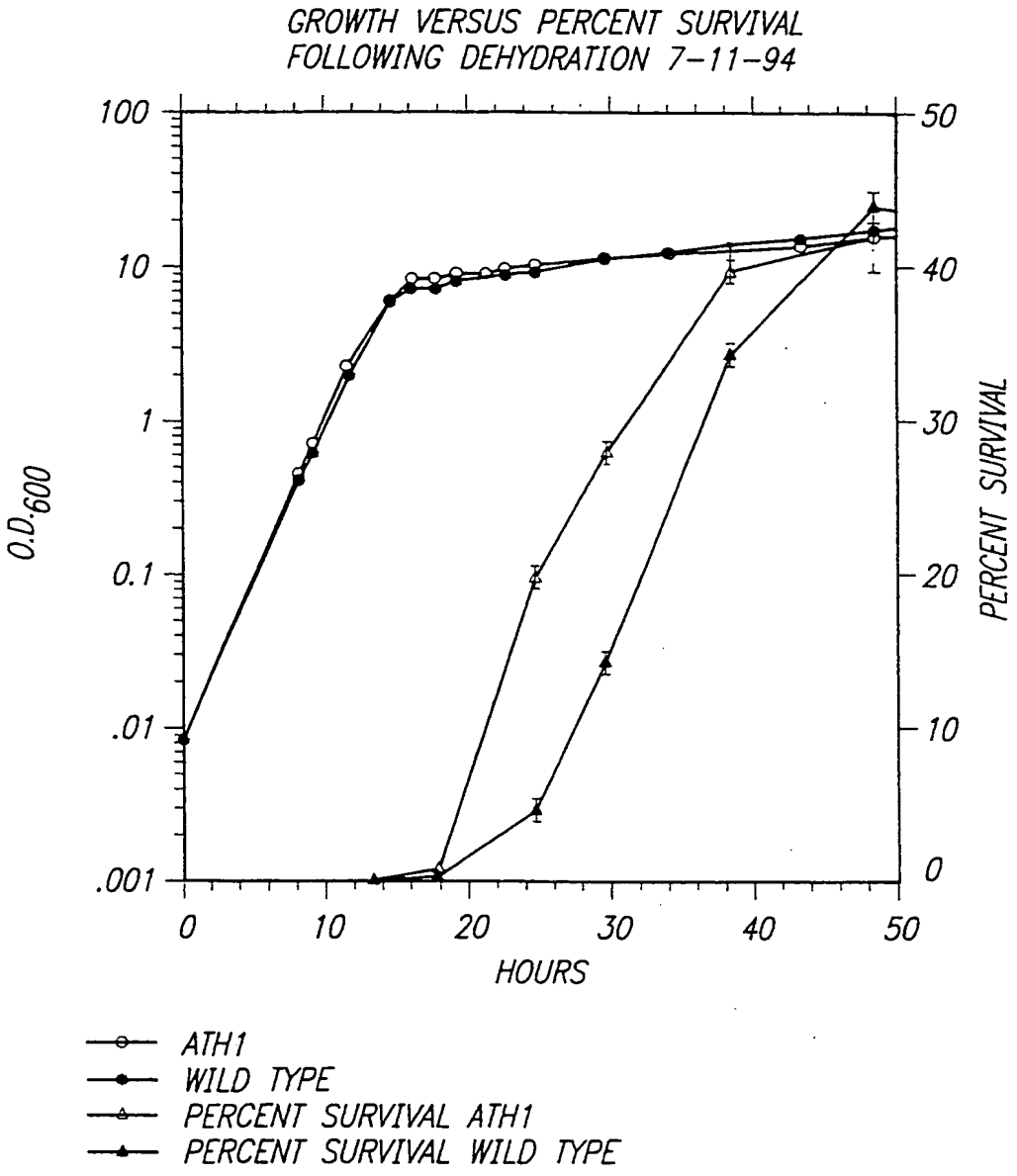


FIG. 2

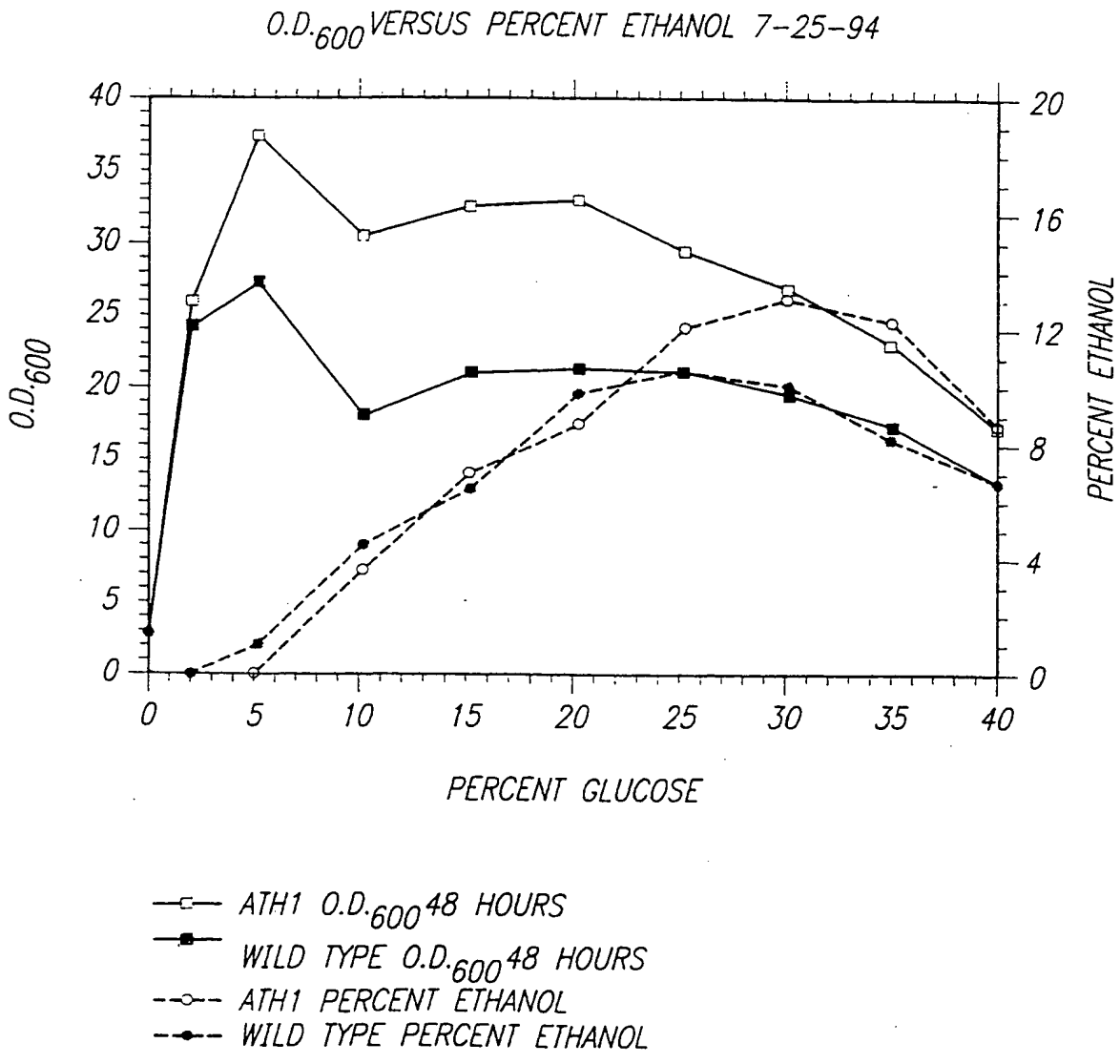
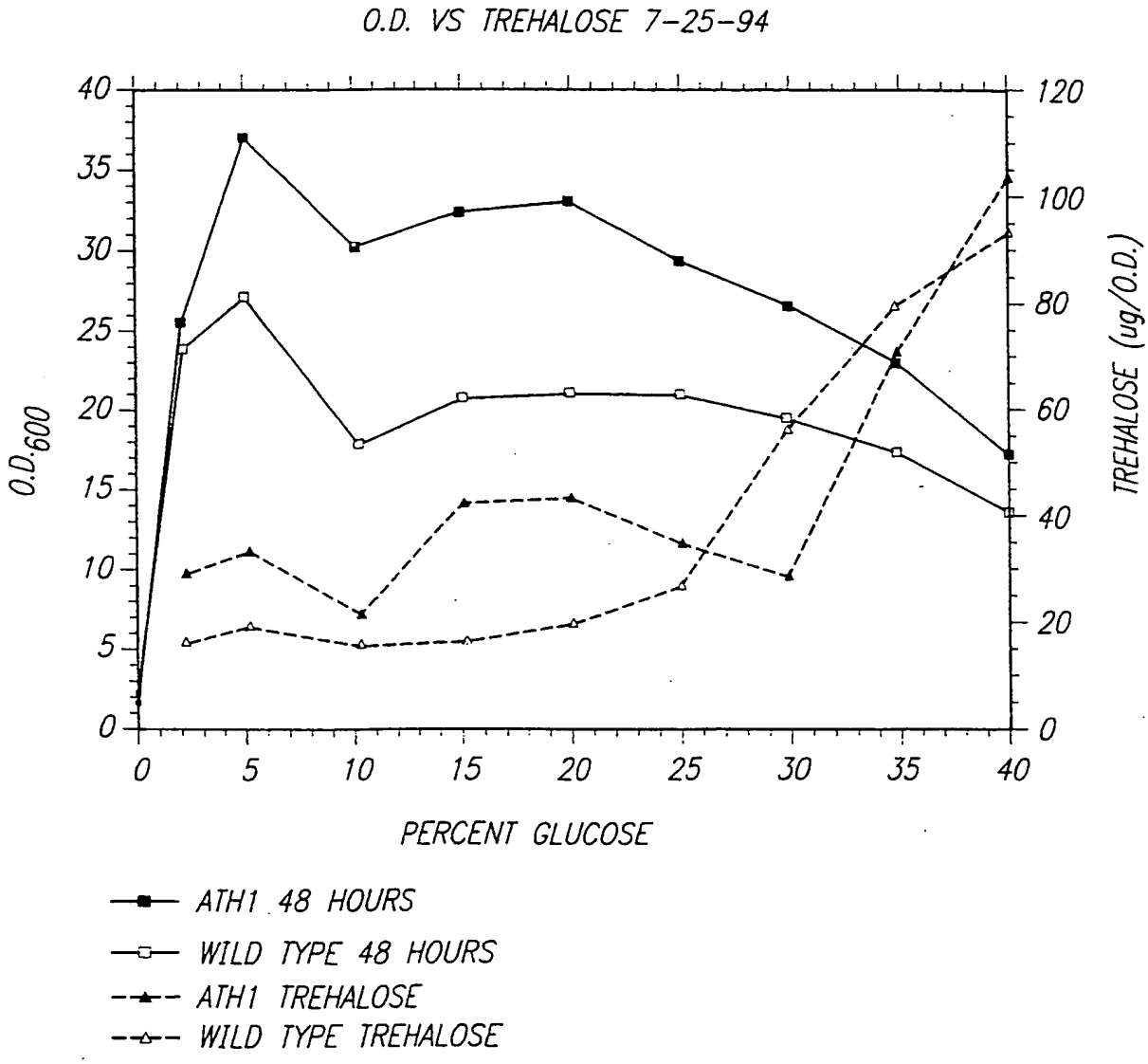


FIG. 3



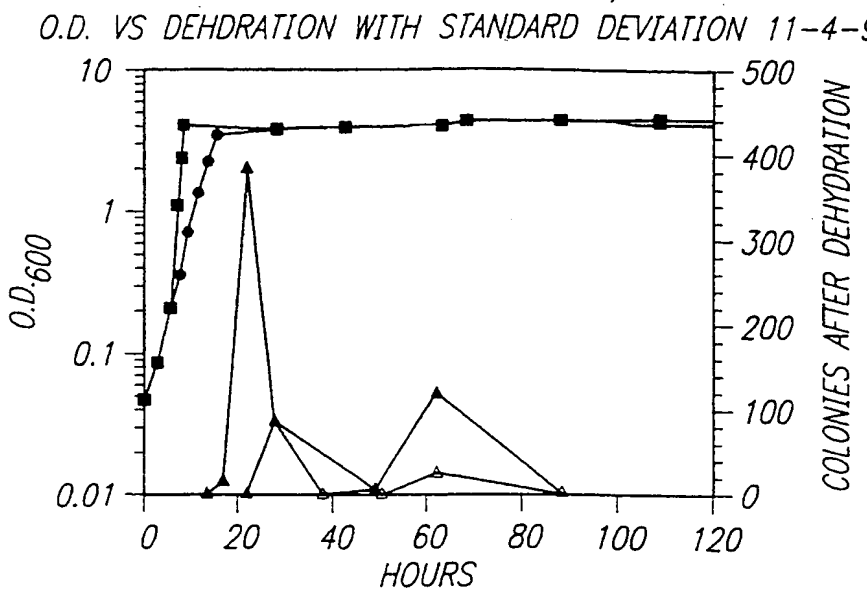


FIG. 4A

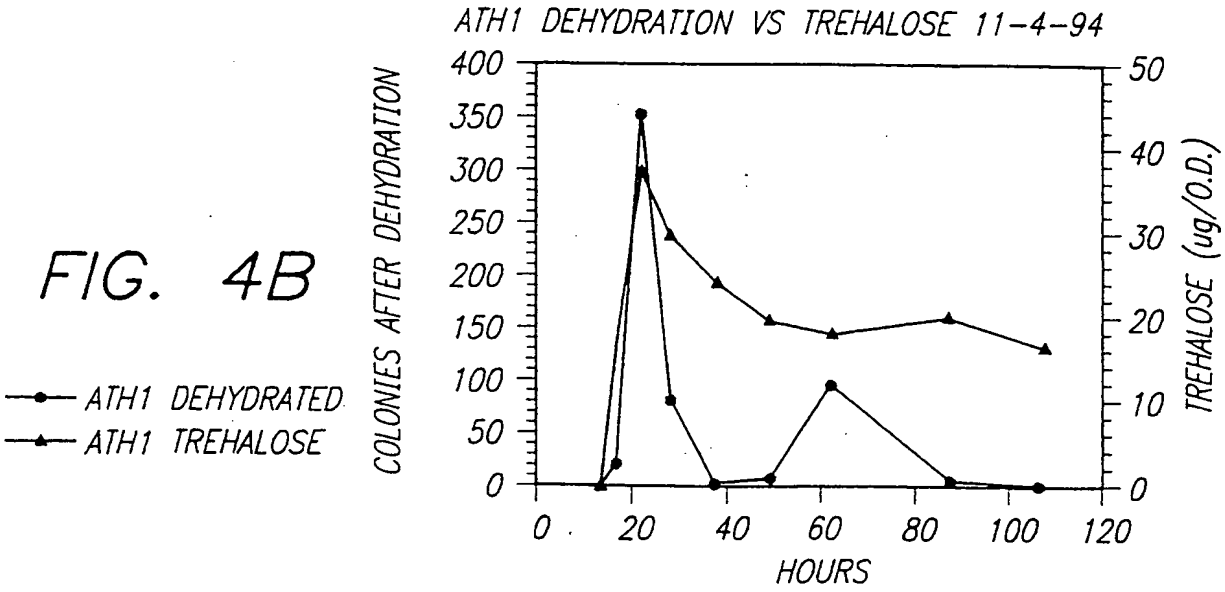


FIG. 4B

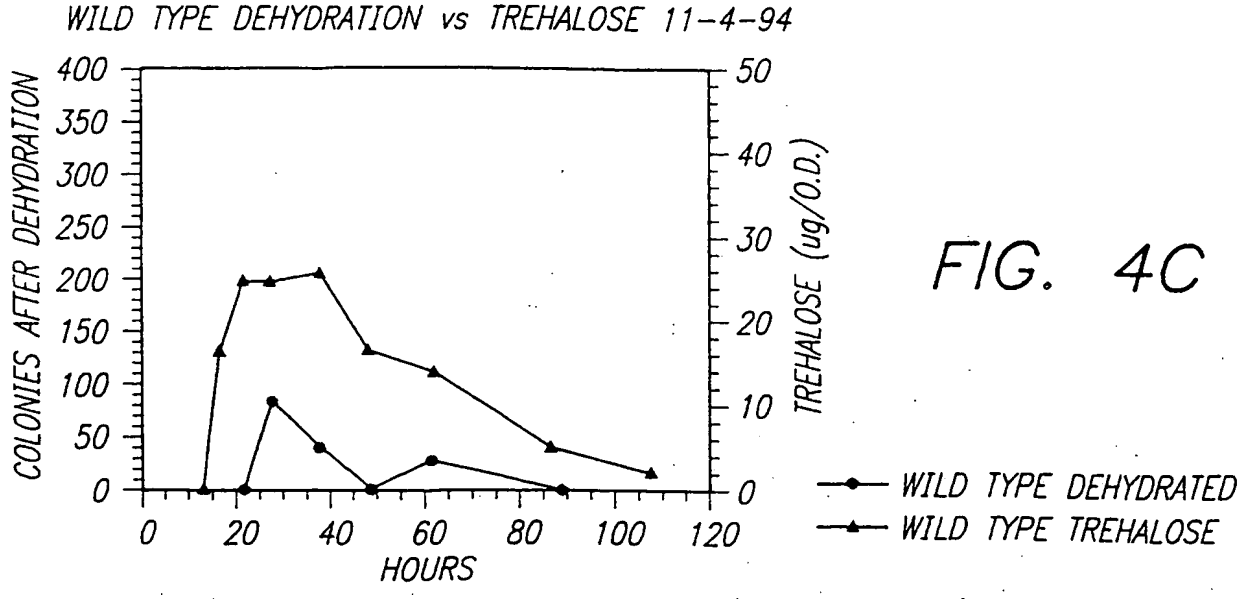


FIG. 4C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10782

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS Letters, Volume 360, issued 1995, Nwaka et al., "Phenotypic features of trehalase mutants in Saccharomyces cerevisiae", pages 286-290, see entire document.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
30 JULY 1996

Date of mailing of the international search report
17 SEP 1996

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Box PCT
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10782

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 1/15, 15/01, 15/31

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 254.2, 254.21; 536/23.1

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/6, 254.2, 254.21; 536/23.1

B. FIELDS SEARCHED
Documentation other than minimum documentation that are included in the fields searched:

NONE

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, APS